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Coexpression of B7-1 and Viral ("Self") Transgenes in Pancreatic β Cells Can Break Peripheral Ignorance and Lead to Spontaneous Autoimmune Diabetes

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Summary

We evaluated the role of the costimulatory molecule B7-1 in overcoming peripheral ignorance in transgenic mice, which expressed the glycoprotein (GP) or nucleoprotein (NP) of lymphocytic choriomeningitis virus (LCMV) as the self-antigen in pancreatic β cells. The viral transgenes or B7-1 alone did not induce autoimmune diabetes (IDDM). However, in bigenic mice expressing B7-1 and LCMV-GP, anti-self (viral) cytotoxic T lymphocytes (CTL) were activated without viral infection and spontaneous IDDM occurred. In contrast, bigenic RIP-B7-1 × RIP-NP mice with thymic expression of the self (viral-NP) antigen deleted the majority of their autoreactive CTL and did not develop spontaneous IDDM. However, these mice developed fast-onset IDDM 14 days after LCMV infection, whereas single-transgenic RIP-NP littermates developed IDDM only within 4–5 months. Rapid IDDM was associated with increased numbers of anti-self CTL and a predominance of IFNγ produced by islet-infiltrating lymphocytes, whereas single transgenic RIP-NP littermates with slow-onset IDDM displayed less anti-self CTL and more IL-4- and IL-10-producing T lymphocytes in pancreatic infiltrates.

Introduction

Destruction of insulin-producing β cells of the islets of Langerhans leads to insulin-dependent diabetes mellitus (IDDM). However, the etiology of autoimmune type I IDDM is not clear (Baekkeskov and Hansen, 1990; Eisenbarth, 1986). First, the self-antigen recognized by anti-self effector cells is unknown. Second, the potential costimulatory molecules that enhance the breaking of peripheral ignorance are unclear; and last, the effector cell(s), molecule(s), or both involved in β cell injury are controversial. One hypothesis postulates a two-event sequence: first, contact with a virus early in life results in persistence of viral antigen in the islets of Langerhans, causing the organism to be ignorant to this viral (self) gene that is located and expressed in the target cell. The second event is an infection with the same virus or an antigenically related pathogen later in life, which induces an immune response to the infecting virus that also cross-reacts with viral (self) antigens in the pancreatic β cells, leading to their destruction and IDDM. Likewise, it is possible that the first event is the expression of a self-protein that shares cross-reactive antigenic determinants or epitopes with a virus. This hypothesis is supported by development of the RIP–LCMV transgenic mouse model and its subsequent manipulation with viral infection (Oldstone et al., 1991; Ohashi et al., 1991; von Herrath et al., 1994a). In these transgenic mice the viral gene (nucleoprotein [NP] or glycoprotein [GP] of lymphocytic choriomeningitis virus [LCMV]) that is inserted into the germline and expressed in pancreatic β cells is essentially a self antigen. However, peripheral ignorance to the transgene can be broken, since upon infection with LCMV in adulthood more than 95% of these transgenic mice generate an anti-viral (anti-self) $CD8^+$ cytotoxic T lymphocyte (CTL) response that leads to IDDM (Oldstone et al., 1991; Ohashi et al., 1991; von Herrath et al., 1994a). Our recent studies (von Herrath et al., 1994a) have shown that RIP–LCMV ($H\text{-}2^b$) mice expressing the transgene only in their β cells do not delete any of their anti-self (viral transgene-specific) CTL and develop fast-onset IDDM within 14 days after LCMV infection. In contrast, RIP–LCMV mice that express the transgene in both their β cells and also in their thymus show a specific reduction of the generation of anti-self (viral transgene-specific) CTL activity (von Herrath et al., 1994a, 1994b). The reason for this reduction is that most anti-self (viral) CTL are deleted in the thymus in RIP–LCMV $H\text{-}2^b$ transgenic mice. In RIP–LCMV $H\text{-}2^d$ transgenic mice only high affinity CTL are deleted in the thymus and anti-self (viral) CTL with lower affinity are still found in the periphery (von Herrath et al., 1994b). While fast-onset IDDM in RIP–LCMV transgenic mice without thymic transgenic expression is dependent only on $CD8^+$ CTL, slow-onset IDDM in RIP–LCMV transgenic mice with thymic expression of the viral (self) antigen depends on both $CD4^+$ and $CD8^+$ T lymphocytes (von Herrath et al., 1994a).

Activation of naive T lymphocytes is a multifaceted process probably requiring at least two signals. The first is the interaction of the T cell receptor (TCR) with the appropriate major histocompatibility complex (MHC)–peptide complex. The second is costimulation that can be provided by B7-1 and B7-2 (Jenkins, 1994; Sagerstrom et al., 1993; Chiodetti and Schwartz, 1992) or cytokines, or both (Sagertrom et al., 1993; Chiodetti and Schwartz, 1992). The B7-1 and B7-2 molecules are related to the immunoglobulin gene superfamily, and both molecules can be expressed on the surface of professional antigen-presenting cells (APCs), such as activated B cells, macrophages, and

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dendritic cells (June et al., 1994). Interaction of B7 with the CD28 ligand provides T cells with a costimulatory signal. In vitro studies have documented that signaling by the TCR alone is not sufficient to induce proliferation and interleukin-2 (IL-2) production by $CD4^+$ T cells (Chiodetti and Schwartz, 1992; Allison, 1994; Harding et al., 1992). TCR–MHC interaction alone not only fails to activate T cells, but TCR stimulation in the absence of costimulation can lead to anergy or cell death (Allison, 1994; Harding et al., 1992; Chen and Nabavi, 1994). Moreover, CTL activation is possible in the absence of most T-helper factors if costimulation by B7–CD28 interaction is provided (Harding and Allison, 1993, Gajewski et al., 1995).

Several animal models have been used to analyze the role of B7 for T cell activation in vivo. Absence of B7-dependent responses has been documented in CD28-deficient mice (Green et al., 1994), and blocking of B7–CD28 interactions can induce tolerance (Boussiotis et al., 1994). When costimulation is blocked by either anti-B7-1 or anti-B7-2 antibodies, the incidence of autoimmune diseases can be differentially influenced (Kuchroo et al., 1995; Lenschow et al., 1995) and opposite effects on the development of T cells into T-helper 1 (Th1) or Th2 phenotypes have been demonstrated (Freeman et al., 1995). However, the different functions of B7-1 and B7-2 in T cell activation and their implication in autoimmunity are not yet fully understood (Harlan et al., 1995).

We and others have generated transgenic mice that express B7-1 locally in pancreatic β cells (Guerder et al., 1994a; Harlan et al., 1994). In these RIP–B7-1 transgenic mice, expression of B7-1 alone does not lead to breaking of ignorance to potential islet–cell autoantigens unless other factors are provided. Accordingly, coexpression of MHC class II (I^e) or tumor necrosis factor-α on islet cells together with B7-1 provides sufficient additional signals to induce autoimmune diabetes in double-transgenic mouse models (Guerder et al., 1994a, 1994b). Further, Harlan and his colleagues (1994) showed that transgenic mice expressing B7-1 and LCMV–GP in pancreatic β cells develop spontaneous IDDM, but only if more than 90% of their circulating T cells were transgenic for a GP-specific TCR. Thus, B7-1 may transform nonprofessional APCs (Nickoloff and Turka, 1994) such as β cells, into competent APCs, although other factors, such as high numbers of potentially autoreactive T cells (Harlan et al., 1994), inflammatory cytokines (Guerder et al., 1994a), or MHC up-regulation are important (Guerder et al., 1994b).

To explore the potential role of B7-1 in breaking immunological ignorance to a viral transgene in the absence of additional inflammatory factors or high numbers of autoreactive transgenic T cells, we generated double-transgenic mice that expressed the viral (self) transgene and B7-1 locally in β cells. This allowed us to address several questions. First, can B7-1 expressed together with a viral (self) gene spontaneously activate an anti-self CTL response and initiate IDDM during the normal lifespan of the animal? Second, does the local expression of B7-1 accelerate the development of IDDM after infection with a virus expressing the same protein as the transgene? Third, if B7-1 enhances autoimmunity, what is the mechanism? Results presented here show, in contrast with the report by Harlan et al. (1994), that the local expression of B7-1 breaks the unresponsive state to the viral transgene as documented by the spontaneous generation of anti-self (viral) $CD8^+$ CTL. Such double-transgenic mice develop IDDM with infiltrating $CD4^+$,$CD8^+$, and B lymphocytes accompanied by an up-regulation of MHC class I and II molecules in the islets of Langerhans. When the double-transgenic mice are infected with LCMV, B7-1 transforms the slow-onset IDDM (occuring 3–6 months postinfection) into a fast-onset IDDM (2–4 weeks after viral infection). Production of Th2 cytokines is markedly reduced and replaced by a Th1 cytokine profile in islets from double-transgenic mice with fast-onset IDDM as compared with islets of single-transgenic mice with slow-onset IDDM. Precursor frequency analysis of autoreactive CTL indicates an amplification of low numbers of anti-self CTL through costimulation by B7-1 and the increased numbers of autoreactive (anti-viral) CTL are directly associated with an enhanced onset of IDDM. Thus, B7-1, when expressed in a local microenvironment, is associated with a shift in cytokine production from Th2 to Th1 and with an increased production of autoreactive lymphocytes that correlates with enhancement or acceleration of autoimmunity.

Results

RIP-GP and RIP-NP Single Transgenic Mice Have Peripheral CTL that Can Be Activated In Vitro to React with the GP or NP Transgene Product

When splenic lymphocytes obtained from RIP–GP 34-20 transgenic mice were incubated with Drosophila melanogaster cells expressing the D^b MHC class I molecule that were coated with LCMV–GP2 peptide (amino acid residues 276–286), they became activated and were able to lyse specifically H-2^b target cells infected with LCMV or coated with the GP2 peptide (Table 1). Such lymphocytes were unable to lyse H-2^b targets coated with the D^b-restricted LCMV–NP peptide (amino acid residues 396–404) or H-2^d targets infected with LCMV. These results were observed in multiple experiments, with two representative experiments shown in Table 1. Similarly, splenic lymphocytes obtained from H-2^b RIP–NP 25-3 mice could be activated to lyse syngeneic but not allogeneic target cells coated with the D^b-restricted NP peptide (data not shown). In contrast with results obtained with lymphocytes from RIP–GP mice, activation of lymphocytes from RIP–NP mice was less frequent (three positive results in a total of seven experiments with RIP–NP mice compared with 6 of 6 with RIP–GP mice). Likely this is the case, because there are larger numbers of potentially autoreactive lymphocytes in the periphery of RIP–GP 34-20 mice than in RIP–NP 25-3 mice, due to expression of the transgene in the thymus in RIP–NP mice (von Herrath et al., 1994a), an observation that was confirmed by precursor frequency analysis displayed in Tables 2 and 3. Thus, peripheral ignorance exists in RIP–LCMV transgenic mice. These observations extend earlier results (Oldstone et al., 1991;

Table 1. Generation of Primary LCMV CTL from Splenic Lymphocytes of RIP GP34-20 Mice by In Vitro Priming

Effector CTLs assayed	Drosphilia Cells (D^b) incubated with		E:T ratio	Percent ^{51}Cr Released from		
	D^b GP2 Peptide	H-2^b Spl Lymphocytes		D^b Targets Coated with		H-2^d target
				No GP2	GP2 peptide	LCMV
Day 7 P^o Spl CTL H-2^b			100:1	0	[20]	0
Day 7 P^o Spl CTL H-2^d			100:1	0	0	[58]
D^b GP2 CTL clone 77–82			5:1	0	[73]	2
Experiment #1	+	Nontransgenics	100:1	0	[28]	7
			50:1	0	5	ND
	+	RIP GP34-20	100:1	0	[32]	5
		transgenics	50:1	0	4	1
	No	RIP GP34-20	100:1	0	0	2
		transgenics	50:1	0	0	ND
Experiment #2	+	Nontransgenics	50:1	0	[57]	3
			25:1	0	[21]	ND
	+	RIP GP34-20	50:1	0	[18]	4
		transgenics	25:1	0	5	ND
	No	RIP GP34-20	50:1	0	0	0
		transgenics	25:1	0	0	ND

CTL activity was determined as described in Experimental Procedures. D^b target cells were RMAS cells coated with the LCMV-GP2 peptide. Effector cells were either splenocytes harvested from nontransgenic mice 7 days after infection with 1 × 10^5 pfu LCMV intraperitoneally, LCMV-GP2-specific CTL clone 77–82, or splenocytes recovered from nontransgenic or RIP-GP transgenic mice and stimulated for 6 days in vitro in the presence of Drosophila melanogaster cells expressing the D^b molecule and coated with GP2 peptide. The in vitro stimulation is described in Experimental Procedures. Boxed areas record significant lysis of the target. ND, not determined.

von Herrath et al., 1994a) showing that LCMV infection generated an anti-self (GP or NP) CTL response, breaking this peripheral ignorance. Fly cells are probably able to activate naive unprimed CTL in vitro, because MHC–peptide density is extremely high on their surface.

Coexpression of B7-1 and Viral GP in Pancreatic β Cells Induces an Anti-Self (Viral) CTL Response In Vivo that Leads to Spontaneous Autoimmune Diabetes

Figure 1 (group A) shows the development of spontaneous IDDM in the absence of LCMV infection in RIP-B7-1 ×

Table 2. Breaking of Peripheral Ignorance through Generation of Anti-Self (Viral) CTL in H-2^b RIP-B7-1 × RIP-LCMV Transgenic Mice following Viral Infection: Injected with LCMV

Transgene(s) expressed in β cells	Thymic expression of LCMV-NP	Days post LCMV infection	Percent ^{51}Cr release from target cells H-2^b				H-2^d	Number of precursors of anti-self CTL		
			LCMV	vv/GP	vv/NP	$NP_{aa396-404}$	LCMV	to GP	to NP	IDDM
None	No	7	40 ± 9	40 ± 6	28 ± 6	40 ± 10	6 ± 2	1:270	1:420	No
RIP-B7-1	No	7	38 ± 6	29 ± 9	37 ± 6	50 ± 6	0	1:300	1:380	No
RIP-GP	No	7	50 ± 9	20 ± 3	26 ± 1	35 ± 8	0	1:400	1:500	No
RIP-GP × B7-1	No	7	52 ± 7	20 ± 4	22 ± 3	38 ± 12	2 ± 2	1:350	1:480	No
RIP-NP	Yes	7	42 ± 6	24 ± 4	6 ± 4	8 ± 3	1 ± 1	1:350	1:5,000	No
RIP-NP × B7-1	Yes	7	41 ± 8	33 ± 9	9 ± 6	10 ± 6	3 ± 2	1:250	1:3,000	No
None	No	28	50 ± 10	39 ± 5	48 ± 8	55 ± 12	5 ± 2	1:2,500	1:2,900	No
RIP-B7-1	No	28	75 ± 8	20 ± 2	44 ± 4	54 ± 2	0	1:1,900	1:2,300	No
RIP-GP	No	28	70 ± 8	35 ± 1	40 ± 3	53 ± 2	4 ± 1	1:2,000	1:3,000	Yes
RIP-GP × B7-1	No	28	35 ± 12	29 ± 4	29 ± 8	50 ± 3	1 ± 1	1:1,260	1:2,900	Yes
RIP-NP	Yes	28	62 ± 6	54 ± 9	8 ± 3	13 ± 7	5 ± 3	1:2,300	1:20,000	No
RIP-NP	Yes	45	52 ± 8	34 ± 3	9 ± 2	11 ± 4	4 ± 2	1:3,500	1:15,000	No
RIP-NP	Yes	140	41 ± 3	53 ± 5	16 ± 3	20 ± 7	3 ± 3	1:2,800	1:10,000	Yes
RIP-NP × B7-1	Yes	14	38 ± 5	18 ± 2	22 ± 6	22 ± 5	0	1:2,560	1:5,340	Yes

CTL activity was measured in RIP-LCMV × RIP-B7-1 mice at various times after challenge with 1 × 10^5 pfu of LCMV intraperitoneally. For assessment of primary CTL activities at day 7 post LCMV infection, single cell suspensions of spleen lymphocytes were used at effector to target (E:T) ratios of 50:1 and 25:1 with data for 50:1 shown. For later timepoints postinfection (day 14 or later), CTL activity was determined from splenic lymphocytes harvested 14, 28, 45, or 140 days after viral challenge and placed in culture for 5 days (see Experimental Procedures). Transgenic mice selected for such analysis of secondary CTL either had normal blood glucose (180 ± 20 mg/dl) or elevated blood glucose (>300 mg/dl). Secondary CTL cultures were used at E:T ratios of 5:1 or 2.5:1 with data for 5:1 shown. Target cells used in all assays were BALB/CI7 (H-2^d) and MC57 (H-2^b) fibroblasts infected with LCMV or recombinant vaccinia viruses expressing NP or GP of LCMV or no LCMV proteins (vv/sc11) or uninfected target cells coated with LCMV-NP peptide (amino acids 396–404). All samples were run in triplicate and variance was <10%. For precursor frequency analysis, H-2^b MC57 fibroblasts (target cells) were infected with vv/GP or vv/NP as reported (von Herrath et al., 1994b). Variation of individual precursor frequencies between experiments was 10–30%. ND, not determined.

Table 3. Breaking of Peripheral Ignorance through Generation of Anti-Self (Viral) CTL in H-2^b RIP-B7-1 × RIP-LCMV Transgenic Mice following Viral Infection: Injected with vv/LCMV Recombinants

Transgene(s) expressed in β cells	Thymic expression of LCMV-NP	7 days post vv/LCMV infection	Percent ^{51}Cr release from target cells H-2^b				H-2^d	Number of precursors of anti-self CTL		IDDM day 28 post-injection
			LCMV	vv/GP	vv/NP	vv/SC11	LCMV	to GP	to NP	
None or RIP-B7	No	vv/GP	19 ± 3	23 ± 4	9 ± 2	10 ± 3	2 ± 3	1:5,000	1:50,000	No
None or RIP-B7	No	vv/NP	18 ± 5	11 ± 2	28 ± 10	11 ± 4	4 ± 3	1:50,000	1:6,000	No
RIP-GP	No	vv/GP	2 ± 2	13 ± 2	12 ± 2	10 ± 3	0	1:7,390	1:50,000	No
RIP-GP × B7-1	No	vv/GP	18 ± 2	25 ± 2	7 ± 3	11 ± 4	2 ± 1	1:1,000	1:50,000	Yes
RIP-GP × B7-1	No	vv/NP	20 ± 3	9 ± 4	25 ± 6	9 ± 8	0	1:50,000	1:5,580	No
RIP-NP	Yes	vv/NP	3 ± 1	9 ± 2	12 ± 3	11 ± 2	0	1:50,000	1:50,000	No
RIP-NP × B7-1	Yes	vv/NP	19 ± 4	11 ± 3	22 ± 4	9 ± 2	0	1:50,000	1:5,000	Yes
RIP-NP × B7-1	Yes	vv/GP	10 ± 4	27 ± 5	8 ± 3	9 ± 4	4 ± 2	1:4,900	1:50,000	No

CTL activity was measured in RIP-B7-1 × RIP-LCMV mice at various times after challenge with 1 × 10^7 pfu vv/GP or vv/NP intraperitoneally. CTL activity was assayed at 7 days (d7) following virus infection. Single cell suspensions of spleen lymphocytes were used at E:T ratios of 50:1 and 25:1 with data for 50:1 shown. Precursor frequency and CTL activity analysis was performed as described for Table 2.

Data shown were obtained in the same experiment. Data from one representative experiment are displayed. Results were confirmed in three other independent experiments.

RIP-GP double-transgenic mice by 8 months of age. This occurs in 40% of such mice. In contrast, spontaneous IDDM did not arise over a 12 month observation period in single-transgenic littermates expressing either the LCMV proteins or the B7-1 molecule alone, nor in double-transgenic mice expressing LCMV-NP and B7-1.

All double-transgenic RIP-B7-1 × RIP-GP mice developing spontaneous IDDM had inflammatory infiltrates in their islets of Langerhans, whereas neither single-transgenic nor double-transgenic mice that had not developed IDDM had such infiltrates (Figure 1, panel A compared with panel B). Infiltrates in the islets were composed of CD4$^+$ and CD8$^+$ T lymphocytes and B cells. Some F4/80-positive macrophages were noted to be located towards the periphery of the islets (data not shown). Expression of MHC class I and II molecules was enhanced in the area of the infiltrated islets. These histological findings resemble those of our previous report (von Herrath et al., 1994a) with RIP-NP or RIP-GP transgenic mice after LCMV infection.

Next, we tested whether pancreas or spleen of double-transgenic mice with IDDM contained LCMV-GP-specific CTL. As shown in Table 4, CTL specific for LCMV GP but not NP were found in spleens and pancreatic infiltrates of uninfected RIP-B7-1 × RIP-GP double-transgenic mice developing spontaneous IDDM. Littermates without such infiltrates or without IDDM did not have LCMV-specific CTL (data not shown). Precursor frequencies of anti-self (viral GP-specific) CTL in unprimed RIP-GP transgenic mice without IDDM were lower than 1:50,000 (Table 4). In contrast, precursor numbers of anti-self CTL were at least 8-fold higher (1:6,000) in RIP-B7-1 × RIP-GP transgenic mice with IDDM. IDDM, spontaneous CTL, or both were not detected in double-transgenic mice expressing LCMV-NP and B7-1 and precursors of anti-self CTL were not found (frequency < 1:50,000; Table 4). Therefore, spontaneous IDDM was associated with the specific in vivo priming and increased frequencies of anti-self (viral) CTL.

Polymerase chain reaction (PCR) analysis showed expression of LCMV-NP RNA in the pancreas and thymus of RIP-B7-1 × RIP-NP mice, whereas RIP-B7-1 × RIP-GP mice expressed the transgene only in the pancreas (data not shown). The lack of spontaneous IDDM in RIP-B7-1 × RIP-NP transgenic mice likely results from the partial elimination of anti-self (LCMV-NP specific) CTL in these mice via negative selection in the thymus, as reported for RIP-NP single-transgenic mice (von Herrath et al., 1994a). The outcome is that precursor frequencies of anti-self CTL (Table 2) are lower in RIP-NP mice.

Virus-Induced Slow-Onset IDDM Is Accelerated by Pancreatic Expression of B7-1 in RIP-NP Transgenic Mice and Develops Independently of CD4$^+$ Cells

We have previously shown that both RIP-NP and RIP-GP transgenic mice develop IDDM following LCMV infection (Oldstone et al., 1991; von Herrath et al., 1994a). In RIP-GP mice IDDM occurs rapidly with a 90% incidence at 1 month following infection (Figure 1, group B). Coexpression of B7-1 and GP on islet cells only marginally accelerated the virus-induced IDDM. In contrast, the coexpression of B7-1 and NP in β cells transformed the usual slow-onset (3–6 months) into fast-onset IDDM (2 weeks). Hence, by 2 weeks after LCMV infection, 100% of RIP-B7-1 × RIP-NP double-transgenic mice developed IDDM, whereas none of the infected single-transgenic RIP-NP littermates had developed IDDM at this time. Indeed, it took 6 months for 80% of RIP-NP single-transgenic mice to develop IDDM after LCMV infection (Figure 1, group B). As seen in Figure 1, 3 weeks after LCMV infection, larger infiltrates and greater destruction of islet cells were found in double-transgenic RIP-B7-1 × NP mice (panel D) than in single-transgenic RIP-NP littermates (panel C).

We then examined whether the fast-onset IDDM was associated with breaking of peripheral ignorance and with appearance of NP-specific CTL in RIP-B7-1 × RIP-NP double-transgenic mice. Table 2 shows that 7 days after LCMV infection, RIP-NP double- and single-transgenic mice had no detectable primary anti-self CTL reactivity (<10%) to the LCMV-NP and had low precursor frequencies of anti-self (LCMV-NP) CTL. However, NP-specific CTL were detected at 14 days post LCMV infection in the

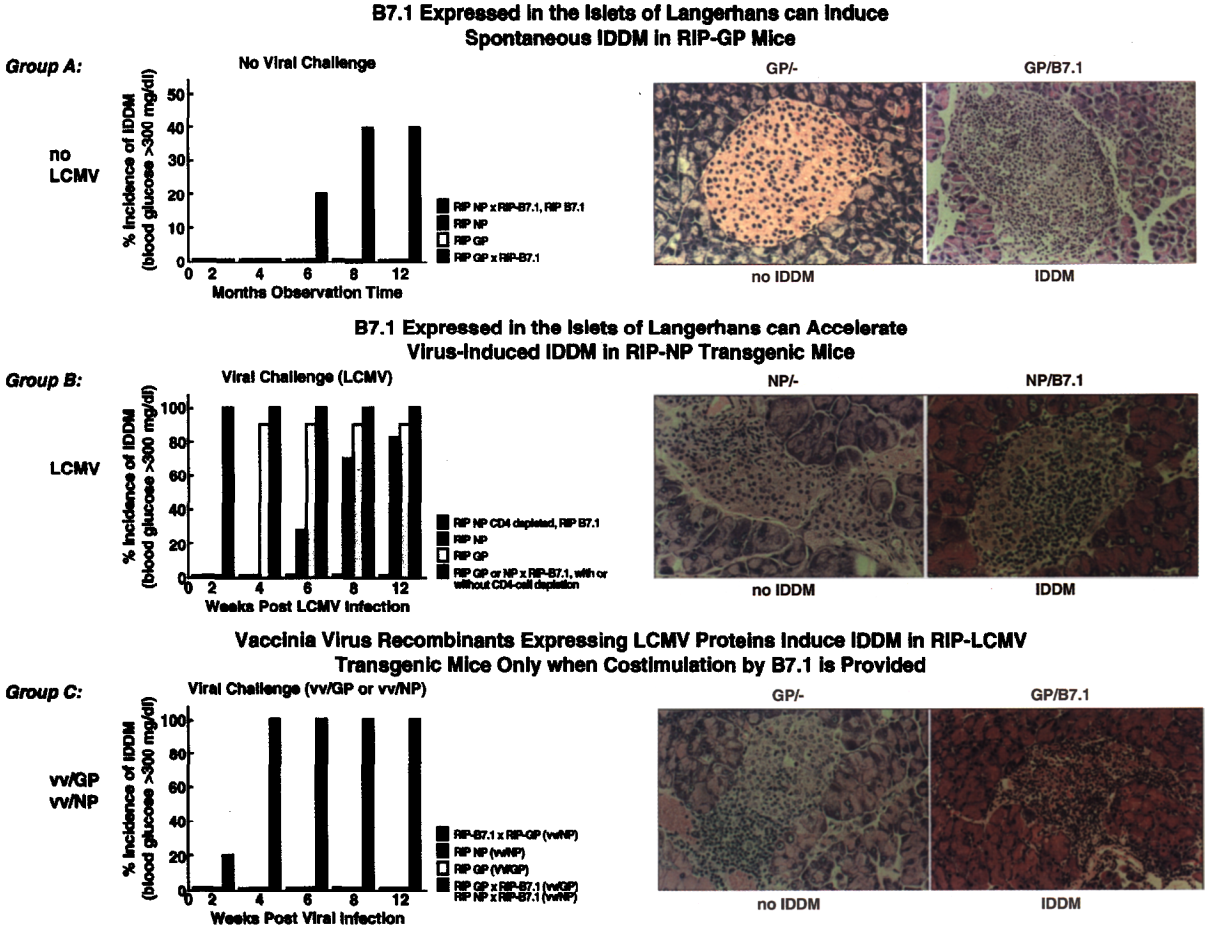

Figure 1. Incidence of Spontaneous IDDM or Virus-Induced IDDM in RIP-B7-1 × RIP LCMV Transgenic Mice

(Group A) Occurrence or lack of spontaneous IDDM (blood glucose > 300 mg/dl, lymphocytic infiltration, and destruction of the β cells of the islets of Langerhans) in single-transgenic RIP-B7-1, RIP-NP, and RIP-GP mice or in double-transgenic RIP-B7-1 × RIP-GP or -NP mice in the absence of LCMV infection is shown. Blood glucose levels were measured at 2 week intervals and 15 mice were analyzed per group. Spontaneous IDDM occurred in 40% of RIP-B7-1 × RIP-GP transgenic mice but not in the other transgenic lines. (A, left) shows a pancreatic islet from a 8-month-old RIP-GP transgenic mouse, while (B, right) shows an islet from a 8-month-old RIP-B7-1 × GP mouse. Neither mouse received an injection of LCMV.

(Group B) Occurrence of IDDM in single-transgenic RIP-NP and RIP-GP mice or in double-transgenic RIP-B7-1 × RIP-GP or -NP mice after infection with 1 × 10^5 pfu LCMV intraperitoneally. Mice were infected at 6–8 weeks of age and were divided into groups with normal CD4 cell counts or groups, where CD4 cells were depleted by antibodies (see Experimental Procedures). $CD4^+$ depletion did not influence the development of IDDM in mice with fast-onset diabetes, but abrogated the development of IDDM in single-transgenic RIP-NP mice. Each group was comprised of 8–10 mice; blood glucose was measured every 2 weeks. (C, left) shows a representative islet of Langerhans from a RIP-NP mouse 3 weeks after challenge with LCMV. A modest degree of infiltration is apparent, blood glucose levels were not elevated. (D, right) shows a representative islet from a double-transgenic RIP-B7-1 × NP mouse 3 weeks after challenge with LCMV. A marked lymphocytic infiltration is observed and was accompanied by a blood sugar of > 500 mg/dl.

(Group C) Incidence of IDDM in single-transgenic RIP-NP and RIP-GP mice or in double-transgenic RIP-B7-1 × RIP-GP or -NP mice after infection with vaccinia virus recombinants expressing LCMV-NP or -GP. Blood glucose levels were measured at 2 week intervals and each group contained 8–10 mice. Vaccinia virus/LCMV recombinants (vv/NP or vv/GP) were injected at a dose of 1 × 10^7 pfu intraperitoneally into 6- to 8-week-old mice. Experimental groups that had an identical time course for the incidence of IDDM are represented together. (E, left) shows an example of a peri-islet infiltration found in a RIP-GP mouse 4 months after vv/GP injection. Only very few cells are found in the islet and blood glucose was 186 mg/dl. (F, right) shows the contrasting picture observed in RIP-B7-1 × GP transgenic mice 1 month after infection with vv/GP. Islets were destroyed, marked infiltration occurred, and blood glucose was > 500 mg/dl. Similar results as those shown in (E) and (F) occurred with RIP-B7-1 × RIP-NP transgenic mice 1 month after infection with vv/NP.

spleen and pancreas of RIP-B7-1 × RIP-NP double-transgenic mice after in vitro stimulation. At this time IDDM was apparent. The frequencies of anti-self CTL were 4-fold higher in RIP-B7-1 × -NP double-transgenic mice (1: 5,340; Table 2) compared with single-transgenic RIP-NP mice (1:20,000; Table 2). Single-transgenic RIP-NP littermates did not have detectable NP-specific CTL activities in the pancreas or spleen on day 14 or even by day 28 postinfection, although some anti-self (viral NP) CTL were found at later stages in prediabetic (day 45) or diabetic mice (day 140). Data from control mice that did not express NP (Table 2) confirmed that a significant reduction in generation of anti-self (LCMV-NP-specific) CTL was detected only in transgenic mice expressing the transgene in the thymus as well as in the islets. NP-specific CTL frequencies at day 7 post LCMV infection were 1:420 in

Table 4. Breaking of Peripheral Ignorance by Spontaneous Generation of Anti-Self (Viral) CTL in Nonvirus-Treated H-2^b RIP-B7-1 × RIP-LCMV Transgenic Mice

Transgene(s) expressed in β cells	Thymic expression of LCMV-NP	Percent ^{51}Cr release from target cells H-2^b				H-2^d	Number of precursors of anti-self CTL		
		LCMV	vv/GP	vv/NP	GP-2$_{peptide}$	LCMV	to GP	to NP	IDDM
None	No	0	0	0	0	0	<1:50,000		No
RIP-B7-1	No	0	0	0	0	0	<1:50,000		No
RIP-GP	No	0	0	0	0	0	<1:50,000		No
RIP-GP × B7-1	No	62 ± 15	34 ± 14	2 ± 1	25 ± 7	0	1:6,000	1:50,000	Yes
		p: 28 ± 9	15 ± 5	5 ± 3	3 ± 2	0	ND		Yes
RIP-NP	Yes	0	0	0	0	0	<1:50,000		No
RIP-NP × B7-1	Yes	0	0	0	0	0	<1:50,000		No

CTL activity was measured in normal nonviral-injected single trangenic RIP-LCMV and RIP-B7-1 mice, as well as doubly transgenic RIP-B7-1 × RIP-LCMV mice. Splenocytes were harvested from 8 to 12-month-old transgenic mice not infected with LCMV. After cultivation for 4–8 weeks on feeder macrophages, splenic lymphocytes were harvested and CTL activity was determined in a 5 hour ^{51}Cr release assay. The effector to target ratios used were 20:1 and 10:1 with data for 10:1 shown. Target cells used were BALB/C17 (H-2^d) and MC57 (H-2^b) fibroblasts infected with LCMV or vaccinia viruses expressing NP (vv/NP) or GP (vv/GP) of LCMV or uninfected cells coated with LCMV-GP2 peptide (amino acids 276–286). In a number of experiments, lymphocytes were harvested directly from the pancreas (p:). All samples were run in triplicate and variance was <10%. For precursors frequency analysis, H-2^b MC57 fibroblasts (target cells) were infected with vv/GP or vv/NP. Precursor numbers from the different groups of transgenic mice shown were obtained in the same experiment. Data from one of three representative experiments are displayed. Similar findings were noted in three independent experiments. Variation of individual precursor frequenceis between experiments was 10–30%. ND, not determined.

non-transgenic mice as compared with 1:5,000 in RIP-NP transgenic mice.

Pancreatic expression of B7-1 circumvented the need for CD4$^+$ help in the virus-induced IDDM of RIP-NP transgenic mice. Virus-induced slow-onset IDDM in RIP-NP H-2^b single-transgenic mice depends on both CD4$^+$ and CD8$^+$ lymphocytes (von Herrath et al., 1994a). In contrast, fast-onset IDDM of RIP-B7-1 × RIP-NP double-transgenic mice that follows LCMV infection requires only the participation of CD8$^+$ lymphocytes, as depletion of CD4$^+$ cells in vivo (Figure 1, group B) did not circumvent the IDDM. CD4$^+$ lymphocytes recovered from transgenic mice with both slow- and fast-onset IDDM proliferated equally well when incubated with LCMV antigen. Specific ^{3}H incorporation by CD4$^+$ T lymphocytes recovered from transgenic mice 40 days after LCMV infection and cultivated with LCMV antigen was 3500 ± 300 cpm for RIP-B7-1 × RIP-NP mice, 3500 ± 500 cpm for RIP-NP mice, 4500 ± 700 cpm for RIP-B7-1 mice, and 320 ± 60 cpm for transgenic mice not infected with LCMV.

Expression of B7-1 In Situ in Islets Enhances Susceptibility to IDDM in RIP-LCMV Transgenic Mice when Only Low Numbers of Anti-Self CTL Are Induced

Our previous experiments (J. Dockter and M. B. A. O., unpublished data) and those of others (Ohashi et al., 1991; Harlan et al., 1994) showed that infection of RIP-LCMV transgenic mice with vaccinia virus recombinants expressing NP or GP of LCMV (vv/GP or vv/NP) failed to induce IDDM and rarely caused islet infiltration, unless an additional inflammatory stimulus such as tumor necrosis factor-α was provided in the islets (Ohashi et al., 1993). Therefore, we performed experiments to examine whether expression of B7-1 in the islets enhanced the susceptibilty to IDDM after infection with vaccinia virus recombinants. All double-transgenic RIP-B7-1 × RIP-LCMV mice manifested IDDM 4 weeks after infection with vaccinia virus recombinants expressing NP or GP (Figure 1, group C), whereas similarly infected single-transgenic (RIP-NP or RIP-GP) littermates did not develop IDDM over a 12-month observation period. The immune response causing IDDM was specific, since inoculation of vv/GP failed to induce IDDM in RIP-B7-1 × RIP-NP transgenic mice and infection with vv/NP did not induce IDDM in RIP-B7-1 × RIP-GP transgenic mice. Although infiltration into the islets was never found 3 months after infection with vv/GP in single-transgenic RIP-GP mice, peri-insulitis was occasionally observed (Figure 1, group C). In contrast, the degree of infiltration was much larger in RIP-B7-1 × RIP-LCMV double-transgenic mice infected with the same vv recombinants (Figure 1, group C). Low levels of anti-self (GP- or NP-specific) CTL activity was found 7 days after infection with vv recombinants in RIP-B7-1 × RIP-LCMV mice, but did not occur in the single-transgenic mice (Table 3). Precursor frequencies of anti-self CTL after infection with vv/LCMV recombinants were up to 10-fold increased in these double-transgenic mice when compared with precursor frequency levels detected in single-transgenic RIP-LCMV mice (Table 3).

Focal Coexpression of B7-1 in β Cells Enhances the Kinetics of Development of a Predominantly Th1-Type Cytokine Profile from the Ordinarily Found Mixed Th1/Th2 Profile Secreted by Lymphocytes Infiltrating Islets of RIP-LCMV transgenic Mice

Differences in Th1 and Th2 responses have been implicated in regulating autoimmunity. To test whether Th1- or Th2- type responses predominated in islets of transgenic mice with fast-onset IDDM (B7-1 expressed in islets) compared with those with slow-onset (no B7-1 expressed in islets), production of Th1 cytokines, IL-2 and interferon-γ (IFNγ), or Th2 cytokines, IL-4 and IL-10 by infiltrating lymphocytes was assessed. Cytokines were detected immu-

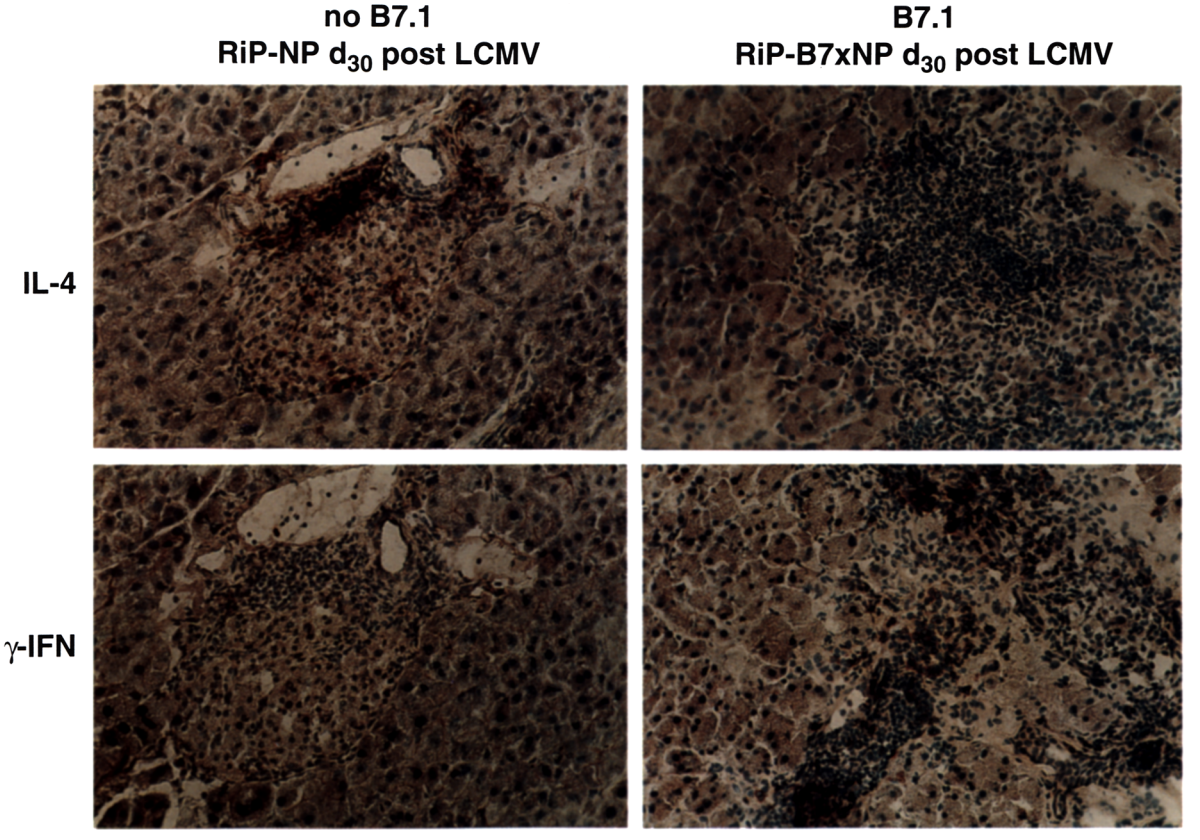

Figure 2. Cytokine-Producing Cells Found in the Pancreas of RIP–B7-1 × RIP–NP and RIP–NP Transgenic Mice

Immunohistochemical staining of 6 μM sections of islets was performed on at least three different sections per mouse; the particular strain and cytokine under observation is indicated in the titles. Representative sections shown were similar for each mouse. Comparable observations were made in at least 7 transgenic mice in two different experimental groups. All sections on display were processed and stained at the same time.

nohistochemically in spleens (data not shown) and infiltrates of RIP–NP and RIP–B7-1 × NP transgenic mice after LCMV infection (Figure 2), but not in normal islets. Production predominantly of IL-4 occurred in single-transgenic RIP–NP mice that had not developed IDDM. In contrast, IFNγ secretion predominated in areas where islets had been infiltrated by $CD4^+$ and $CD8^+$ cells of transgenic mice with B7-1 expression who developed IDDM (Figure 2).

The ELISPOT test was then used to quantitate the numbers of lymphocytes producing the individual cytokines. Both Th1 cytokines (IL-2, IFNγ) and Th2 cytokines (IL-4, IL-10) were produced by activated lymphocytes passing through the pancreas of nontransgenic (data not shown) or RIP–B7-1 transgenic mice after infection with LCMV (Figure 3). Maximal responses were observed on day 7 after infection and declined over time. Similar results were observed with RIP–NP single-transgenic mice before the onset of IDDM. By contrast, RIP–GP mice that develop fast-onset IDDM after LCMV infection demonstrated an almost complete loss of IL-4- and IL-10-producing cells. Similarly, the disappearance of Th2 type cells was observed in RIP–B7-1 × GP and RIP–B7-1 × NP transgenic mice after LCMV infection. For example, numbers of lymphocytes secreting IL-4 and IL-10 were approximately 5-fold lower in RIP–B7-1 × NP double-transgenic mice with IDDM at 14 days postinfection than in RIP–NP mice without IDDM at day 45 postinfection. Moreover, Th1- and not Th2-secreting lymphocytes were found in uninfected diabetic RIP–B7-1 × GP transgenic mice that spontaneously developed diabetes. Interestingly, production of Th1 cytokines appears similar in all cases regardless of the development of IDDM. Thus, while LCMV induces both Th1 and Th2 cytokine production, a marked reduction of Th2-type cytokines (IL-4, IL-10) was found in the pancreas of transgenic mice with IDDM occurring as early as 7 days after LCMV infection in RIP–B7-1 × RIP–LCMV double-transgenic mice or RIP–GP single-transgenic mice.

Discussion

A central step leading to autoimmune diabetes and other autoimmune diseases is the breaking of peripheral ignorance to self-antigens (Miller and Flavell, 1994). The RIP–LCMV transgenic mouse model (Oldstone et al., 1991; Ohashi et al., 1991), which places a known self-antigen (viral protein) in β cells, offers an opportunity to explore the pathogenesis of IDDM. Recent studies have shed light on the role of several cytokines (von Herrath et al., 1995; Lee et al., 1994, 1995; Stewart et al., 1993) as well as the thymus (von Herrath et al., 1994a) in the pathogenesis of IDDM. Here, we analyze the role of the costimulatory

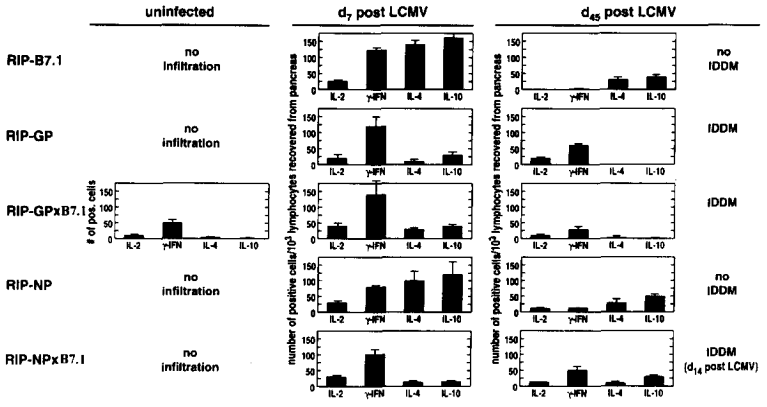

Figure 3. Number of Lymphocytes Producing IL-2, IL-4, IL-10, and IFNγ Recovered from the Pancreas of RIP–B7-1 × RIP–LCMV Transgenic Mice

ELISPOT assays were carried out as described in Experimental Procedures. Number of positive cells for each cytokine is shown in black bars ± 1 SEM. The origin of lymphocytes is indicated on the left side of each panel and preparation is described in Experimental Procedures. Pancreas from uninfected single-transgenic mice did not contain enough lymphocytes to allow analysis. Pancreata from single transgenic RIP–B7-1 mice without infiltration contained few lymphocytes after LCMV infection and cells obtained from 3–4 different pancreata were pooled.

molecule B7-1 in the breaking of peripheral ignorance. We note several interesting and novel findings. First, costimulation by B7-1 provided through in situ expression on islet cells breaks immunologic ignorance to a self (viral) antigen in vivo in otherwise normal unmanipulated transgenic mice. Second, the B7-1 molecule can transform virus-induced slow-onset IDDM that occurs when only low numbers of anti-self (viral) CTL are present, into a fast-onset IDDM. Third, enhancement of autoimmunity in the presence of B7-1 is associated with the amplification of autoreactive CTL and with shifting the local pancreatic cytokine profile from Th2 to Th1.

The data presented here leads us to hypothesize the following scenarios for the induction of autoimmune diabetes. In uninfected transgenic mice potentially autoreactive T cells specific for peripheral self-antigens are present (Table 1) and likely circulate through the organism. In the absence of a viral challenge, these cells remain silent, are not activated, and therefore do not cause disease. However, when otherwise nonprofessional APCs provide costimulation by B7-1 in the islets along with expression of self (viral) antigen, these T cells, when circulating through the pancreas, can become activated and cause IDDM. Despite using inbred strains of mice that express equivalent levels of the LCMV and B7-1 transgenic on their β cells, only 40% of such mice developed IDDM. A possible explanation is that nonactivated anti-self T cells infrequently enter the islets, unless additional inflammatory factors are provided (Lee et al., 1995).

IDDM rarely occurred in RIP–B7-1 single-transgenic mice and less than 2% of such mice developed disease (Guerder et al., 1994a). This indicates that B7-1 alone can not usually break ignorance to self-antigens normally expressed by islet cells. Nevertheless, spontaneous IDDM in RIP–B7-1 × RIP–GP transgenic mice occurred, probably because the viral GP was expressed more abundantly on β cells than other natural self-antigens usually present on islet cells. The likely outcome is generation of increased numbers of self-reactive (viral GP-specific) CTL.

Our finding of spontaneous IDDM in RIP–B7-1 × RIP–GP transgenic mice stands in contrast with the report of Harlan et al. (1994), in which spontaneous activation of potentially anti-self CTL and development of diabetes did not occur unless an abundance of TCR transgenic T cells (>90%) was provided (TCR × RIP–GP × RIP–B7-1 triple-transgenic mice). One possible explanation is that B7-1 expression in RIP–B7-1 transgenic mice used in the Harlan report (1994) was lower than in our RIP–B7-1 transgenic mice. Expression levels of B7-1 as assessed by immunohistochemical analysis were higher in our transgenic mice than levels of B7-1 normally found on APCs. In agreement with our findings, the RIP–B7-1 × GP model of Harlan et al. (1994) supports the concept that development of spontaneous autoimmune disease is dependent on the number of potentially autoreactive T cells present.

Previous studies with the RIP–LCMV transgenic model showed that coexpression of IFNγ in islets together with LCMV proteins led both to enhanced spontaneous autoimmune diabetes and to the generation of anti-self (viral transgenic-specific) CTL (Lee et al., 1995). By contrast, expression of IL-2 and IL-10 together with LCMV transgenes in the pancreas did not lead to spontaneous CTL activation or IDDM (von Herrath et al., 1995; Lee et al., 1994). These findings show that in addition to B7-1, IFNγ can break peripheral ignorance. How this occurs is not clear and whether the mechanism of breaking of peripheral ignorance by B7-1 or IFNγ are similar or different is not yet known.

Constitutive expression of B7-1 on islet cells changed the kinetics of the virus-induced slow-onset IDDM to fast-onset IDDM in RIP–NP H-2^b transgenic mice. Slow-onset IDDM occurred in RIP–NP mice, because most but not all anti-self (NP-specific) CTL had been eliminated by negative selection in the thymus (von Herrath et al., 1994a). Owing to the small numbers of activated anti-self CTL, it is likely that the event of an autoreactive CTL entering the pancreas was less frequent in the slow-onset IDDM model than in the fast-onset IDDM observed in RIP–GP mice, which do not delete their anti-self CTL by negative selection. Precursor frequency analysis (Table 2) showed that only small numbers of anti-self CTL are available to enter the pancreas in RIP–NP mice. The development of IDDM in these mice depends on additional factors provided by CD4$^+$ helper cells (Ohashi et al., 1993, von Herrath et al. 1994a). Local pancreatic expression of IL-2 potentiated

IDDM in these RIP–NP transgenic mice after LCMV priming, but did not eliminate the need for $CD4^+$ help. This suggested that other $CD4^+$-dependent factors might be required (von Herrath et al., 1995). However, when costimulation by B7-1 was provided in situ in RIP–NP transgenic mice, the need for $CD4^+$ help was eliminated (Figure 1), and IDDM developed rapidly after LCMV infection.

Infection with vaccinia virus recombinants expressing NP or GP of LCMV caused IDDM only in double-transgenic RIP–B7-1 × RIP–LCMV mice, but not in single-transgenic RIP–LCMV littermates (Figure 1; Tables 2 and 3). These vaccinia virus recombinants induced LCMV-specific CTL, but in nontransgenic and RIP–LCMV transgenic mice their frequencies were low so that no primary (day 7) CTL response but only a secondary memory CTL response was detected (Tables 2 and 3; Whitton, 1990). In contrast, RIP–B7-1 × RIP–LCMV transgenic mice made a primary LCMV-specific CTL response after infection with vaccinia virus recombinants expressing NP or GP and developed IDDM, likely owing to the local amplification of low numbers of anti-self CTL that entered the pancreas.

Taken in toto, our observations strongly suggest a direct correlation between the development and frequency of autoimmune diabetes with the number of precursors of autoreactive CTL. We propose that a local expansion of anti-self CTL occurred after entering the pancreas and coming into contact with β cells that expressed both viral (self) antigen and B7-1. As a result, large numbers of anti-self CTL were generated locally and led to fast-onset IDDM. Recent experiments indicated that expression of B7-1 not only leads to clonal expansion of CTL but also induced the maturation of precursor CTL into functional effector CTL (S. G. and R. A. F., unpublished data). In addition, it is likely that B7-1 induced a change in the local cytokine milieu from Th2 to Th1 and this increased the production of anti-self CTL.

A direct link between cytokine milieu and the development of autoimmune diabetes was observed (Figures 2, 3). Islets of transgenic mice with fast-onset IDDM and pancreatic expression of B7-1 contained less Th2-type cytokine (IL-4, IL-10)-producing cells than islets from transgenic mice with slow-onset IDDM or nontransgenic spleens at the same time after infection (Figure 3). This supports the concept that constitutive expression of B7-1 can influence the local microenvironment leading to the reduction in numbers of Th2-type T lymphocytes. Recent results from L. Glimcher and her colleagues (Kuchroo et al., 1995) have indicated that blocking B7-1 interactions with antibodies decreases severity of autoimmune disease (EAE), whereas blocking B7-2 interactions increases disease severity. As supported by other studies (Paul and Seder, 1994; Modlin and Nutman, 1993; Scott et al., 1994), these data add to the evidence that predominance of either Th1- or Th2-type cytokines in inflammation may play a role in the regulation of autoimmune disease. Similarly, we found in all cases of spontaneous or virus-induced IDDM that a specific disappearance of Th2-type cytokines resulting in a predominance of Th1 cytokines was associated with the development of IDDM. Thus, therapeutic interventions that successfully maintain a Th2 cytokine profile while inhibiting or replacing a Th1 profile of infiltrating lymphocytes may be helpful in treating IDDM. Studies to test this hypothesis are currently underway.

Experimental Procedures

Transgenic Mouse Lines

The transgenic mouse lines expressing the LCMV–NP (RIP–NP 25-3) and GP (RIP–GP 34-20) were generated in H-2^b (C57BL/6J) mice using the RIP vector and cDNA clones for LCMV–NP and –GP (Oldstone et al., 1991). RIP–GP 34-20 mice expressed the transgene only in their β cells, while RIP–NP 25-3 mice expressed the transgene in both their pancreatic β cells and their thymi, but not in other tissues (von Herrath et al., 1994a). Neither group of transgenic mouse lines displayed any spontaneous mononuclear cellular infiltrate in or around the islets nor did they develop IDDM (blood glucose levels above 300 mg/dl, pancreatic insulin levels below 20 μg insulin per gram of pancreatic tissue) unless challenged with LCMV (Oldstone et al., 1991). Transgenic mice expressing the human B7-1 molecule were generated as reported (Guerder et al., 1994a). These mice were transgenic for the human B7-1 cDNA driven by the RIP and expressed the B7-1 molecule only in the β cells of the pancreas (Guerder et al., 1994a). The RIP–B7-1 transgenic mice had no mononuclear cell infiltrates in or around their islets and fewer than 2% manifested spontaneous IDDM. Doubly transgenic mice that expressed both LCMV–NP or –GP and B7-1 locally in their β cells were made by mating H-2^b RIP–LCMV to RIP–B7-1 mice. Progeny were bred for two generations to the C57BL/6J (H-2^b) background, and expression of LCMV–NP or –GP and B7-1 transgenes was confirmed by slot–blot analysis (Oldstone et al., 1991; Guerder et al., 1994a; Maniatis et al., 1990). For RNA analysis, RNA was extracted from peripheral blood mononuclear cells and organs (thymus and pancreas) using the GTC method (von Herrath et al., 1994a; Maniatis et al., 1990). Expression of LCMV RNA was analyzed by RT–PCR (von Herrath et al, 1994a) run for 40 cycles, after which the products were analyzed on a 3% agarose gel.

Virus

The virus used was the Armstrong (ARM) strain of LCMV, clone 53b. Its origin, quantitation by plaquing, sequence, and biological properties have been described (Dutko and Oldstone, 1983). Vaccinia virus recombinants expressing GP or NP of LCMV were generated and titered as described (Whitton, 1990; Whitton et al., 1988).

CTL Assays and In Vitro Culture of CTL Lines

CTL activity was determined in a 5 hr in vitro ^{51}Cr release assay (Oldstone et al., 1991). Samples were run in triplicate and variance was <10%. To judge CTL activity, uninfected targets or those infected with either LCMV–ARM (MOI 1) or vaccinia virus-expressing LCMV–NP or LCMV–GP (MOI 3) were used. Target cells were H-2^b (MC57) and H-2^d (BALB/c17) fibroblasts. Lymphocytes from spleens of 8- to 12-week-old unimmunized mice or mice inoculated intraperitoneally 7 days earlier with 1×10^5 pfu of LCMV (ARM) or 2×10^7 pfu of vaccinia virus recombinants expressing GP or NP of LCMV (vv/GP or vv/NP) were used as effectors at 50:1, 25:1, and 12:1 effector:target (E:T) ratios, respectively. H-2^b- and H-2^d-restricted LCMV-specific CTL clones (Lewicki et al., 1992) were used at E:T ratios of 10:1, 5:1, and 1:1, respectively. Secondary CTL lines were generated and maintained as described (von Herrath et al., 1994a, 1995; Lee et al., 1995) and used at E:T ratios of 10:1 and 5:1. Uninfected target cells were coated with peptides at 30 μg/well 30 min prior to adding CTL and starting the 5 hr incubation for the ^{51}Cr release assay. To determine CTL activity after secondary stimulation, spleen cells harvested from mice 30–120 days after primary inoculation with 1×10^5 pfu LCMV intraperitoneally were incubated with MHC-matched irradiated LCMV–infected macrophages in the presence of T cell growth factor and irradiated syngeneic spleen feeder cells for 5–10 days.

Precursor Frequency Analysis

For precursor frequency analysis (limiting dilution analysis), spleen cells were harvested on day 7, 28, 60, 90, and 120 after primary LCMV infection. These cells were diluted serially and cultivated in 96-well flat-bottomed plates in the presence of T cell growth factor and synge-

neic irradiated LCMV-infected (10^3 pfu/ml) spleen cells (10^5/well). After 5–9 days, each well was assayed for cytotoxic activity (described above) on target cells that were uninfected, or infected with LCMV or vaccinia viruses and expressed GP or NP. Positive cultures were defined as specific lysis 3 SD above the lysis found in negative controls (3% ± 2% for all experiments shown). The fraction of negative cultures was determined for each dilution (48 cultures total per dilution). Precursor CTL (pCTL) numbers were assessed as follows: The fraction of negative cultures (y axis) was plotted on a semilogarithmic scale against the number of splenocytes per culture (x axis). pCTL frequencies are defined by the slope of the linear regression between at least three separate data points. The formula used was $f = (\ln a - \ln y)/n$; f is the frequency of pCTL; n, the number of splenocytes added to each culture; *a*, the y axis intercept (in our experiments 100% ± 4%); and *y*, the percentage of negative cultures.

Detection of Peripheral Ignorance/In Vitro Induction of CTL

Functional cDNA for the H-2D^b molecule was obtained using a retroviral shuttle vector (Joly and Oldstone, 1991). Drosophila melanogaster cells express large amounts of empty MHC molecules (Jackson et al., 1992; Matsumura et al., 1992) after transfection with the H-2D^b cDNA. Cells were grown in Insect Xpress Medium (Whittaker Bioproducts, Walkersville, Maryland) at 28°C. Expression of D^b molecules was induced using $CuSO_4$ at a final concentration of 0.7 mM and D^b expression was confirmed after 24 hr by FACS analysis using a monoclonal antibody to MHC class I D^b (Joly and Oldstone, 1991). When more than 70% of the transfected Drosophila cells expressed D^b molecules after $CuSO_4$ induction, they were harvested 24 hr later and 1×10^7 fly cells were incubated for 2–3 hr at 28°C in 50 ml of Insect Xpress medium containing LCMV peptides (FQPQNGQFI, LCMV–NP and SGVENPGGYCL, LCMV–GP2) at a final concentration of 200 μM. Following a 1 hr incubation at 37°C, fly cells were washed by centrifugation and resuspended in RPMI containing 10% fetal calf serum (FCS). For in vitro CTL induction, fly cells were incubated with H-2^b splenocytes at 1:50, 1:20, and 1:10 ratios, respectively, for 6 days at 37°C in a humidified 5% CO_2 incubator. CTL assays were performed as described above but using ^{51}Cr-labeled RMAS (H-2^b) cells as target cells that were grown for 36 hr at 28°C in RPMI containing 7% FCS. LCMV–peptides were preincubated at a final concentration of 10 μM with the target cells for 1 hr prior to the CTL assay, and excess amounts of peptides were removed by washing cells three times. Effector splenocytes were harvested from the incubation with the fly cells by centrifugation and used at E:T ratios of 100:1, 50:1, and 25:1, respectively, in the CTL assay.

Proliferation Assays

Spleen cells were harvested 9 or 40 days after LCMV infection of mice. Inguinal lymph nodes were obtained 9 days after animals were primed with purified ultraviolet (UV)-inactivated LCMV inoculated at the base of the tail (von Herrath et al., 1994a). Splenocytes were added to nylon wool columns both to enrich for lymphocytes and to delete APCs (Kasaian and Biron, 1990; Kasaian et al., 1991). APCs consisted of 2×10^5 irradiated syngeneic spleen cells per well. When indicated, purified UV-inactivated LCMV was added at 0.8 μg/ml final concentration. Anti-CD4 and -CD8 antibodies were used at 10 μg/ml and IL-2 at 10 U/ml. The medium was RPMI containing 7% FCS and glutamine. Assays were run for 72 hr using 96-well round-bottomed plates after which tritiated thymidine diluted 1:100 in RPMI was added to each well, and plates were incubated for an additional 16 hr. Thymidine incorporation was determined after harvesting cells from each well as described (Kasaian and Biron, 1990; Kasaian et al., 1991).

Recovery of Pancreatic Lymphocytes

Lymphocytes were isolated from pancreata as described (Oldstone et al., 1991; von Herrath et al., 1995). In brief, tissues obtained from transgenic mice were freed from fat and surrounding lymphoid tissue. After collagenase digestion, lymphoid cells were purified through a Ficoll–Hypaque gradient.

Analysis of IDDM

IDDM was defined as hyperglycemia, low levels of pancreatic insulin, and mononuclear infiltration into the islets. Blood samples were obtained from the retro-orbital plexus of each mouse at weekly, biweekly, or monthly intervals. Amount of glucose in the blood was determined using Accucheck II strips (Boehringer-Mannheim, Indianapolis, Indiana). Mice with glucose values >300 mg/dl were considered diabetic (normal blood glucose for age- and sex-matched controls was mean ± 1 SEM [20–40 mice /group] 185 ± 7mg/dl). Normal levels of insulin were 50 μg insulin/mg of pancreas ± 12 (Oldstone et al., 1991) and levels of 20 μg/mg of insulin or less of pancreatic tissue as determined by radioimmune assay (Oldstone et al., 1991) were considered abnormal. Absence or presence of mononuclear cells in the islets was determined in tissues fixed in 10% zinc formalin, mounted in paraffin, and stained with hematoxylin and eosin (Oldstone et al., 1991; von Herrath et al., 1994a).

Immunohistochemical Analyses of Tissues

Immunochemical studies were carried out on 6 μm cryomicrotome sections. Sections were obtained from tissues quick frozen in OCT compound, fixed for 5 min in 1% paraformaldehyde, washed in phosphate-buffered saline (PBS), and incubated with avidin–biotin to block nonspecific binding. Primary antibodies consisting of rat anti-mouse CD4 (clone RM 4-5), anti-CD8 (clone 53-6.7), anti-B220 (clone RA3 6B2), anti-F4/80 (clone A3-1), anti-MAC-1 (clone M 1/70), anti-class I (clone M 1/42), anti-class II (clone M5/114), anti-B7-2 (clone GL-1), anti-IL-2 (clone S4B6), anti-IL-4 (clone BVD6-24G2), anti-IFNγ (clone XMG1.2) (Pharmingen, San Diego, California; Boehringer Mannheim, Indianapolis, Indiana) were applied for 1 hr. After washing, the secondary antibody, biotinylated goat anti-rat immunoglobulin G (IgG) (Vector Laboratories, Burlingham, California), was added and the color reaction obtained after sequential treatment with an avidin–horseradish peroxidase conjugate (Boehringer-Mannheim, Indianapolis, Indiana) and diaminobenzidine–hydrogen peroxide (von Herrath et al., 1994a). Anitbodies for cytokine staining were described earlier by Abbas and his colleagues (Bogen et al., 1993).

Phenotyping and Selective Depletion of Lymphocytes

Cultured lymphocytes were phenotyped by FACS analysis utilizing monoclonal antibodies to murine CD4 (rat monoclonal antibody YTS 191.1.1) and CD8 (rat monoclonal antibody YTS 169.4.2) (Oldstone et al., 1991). For depletion of CD4$^+$ or CD8$^+$ T lymphocyte subsets, monoclonal rat anti-mouse antibodies 53-6-72 and GK 1.5 were used, respectively (von Herrath et al., 1994a). In brief, mice were injected intravenously with 1 mg/0.2 ml of the appropriate antibody 2 days before and 4 days after LCMV infection. Depletion was confirmed by FACS analysis 7 days after LCMV infection.

Assessment of Cytokine Production by Single Cells

Cytokines (IL-4, IL-10, IL-2, and IFNγ) produced by lymphocytes infiltrating the pancreas or by splenocytes were detected using the ELISPOT (Pharmingen, San Diego, California) assay. In brief, 96-well Millititer HA plates (Millipore, Bedford, Massachusetts) were coated with the respective capture antibodies for IL-2, IL-4, IL-10, and IFNγ diluted 1:200. After overnight incubation at 4°C, plates were washed four times with PBS and preincubated with RPMI containing 10% FCS for 1 hr at 37°C. Suspensions of lymphocytes recovered from pancreata or spleens were added at various dilutions in RPMI, 10% FCS ranging from 1×10^4–1×10^2 cells per well, and plates incubated overnight at 37°C in a humidified incubator at 5% CO_2. The next day, plates were washed three times with PBS and three times with PBS containing 0.05% Tween 20, then the respective detection antibodies for the cytokines were added at 1:200 in PBS/Tween containing 1% FCS. Plates were incubated at 4°C overnight and washed three times in PBS/Tween before avidin–peroxidase conjugate (Boehringer, Indianapolis, Indiana) was added at 1:500 dilution. After a 2 hr incubation at room temperature, plates were washed three times in PBS and the color substrate solution containing 3-amino-9-ethylcarbazole and H_2O_2 was added and left on the plates for 30 min. Plates were then rinsed in water and dried, and spots representing cytokine producing cells were counted using an Olympus SZH stereo-zoom microscope.

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lated by centrifugation (500 × g) at 24°C over 30%/70% discontinuous Percoll (Pharmacia) gradients. Cells collected from the 30%/70% interface were washed and resuspended in 2.4G2 supernatant.

Flow Cytometry

Fluorescein isothiocyanate (FITC)-conjugated MAbs to CD3 (T cells) and B220 (B cells) were purchased from Pharmingen (La Jolla, California). FITC-anti-F4/80 (macrophage/microglial cells) was purchased from Caltag (South San Francisco, California). Purified anti-B7-1 (clone 16-10A1) and anti-B7-2 (GL-1) MAbs were biotinylated using NHS-LC-biotin (Pierce, Rockford, Illinois). Avidin-R-phycoerythrin (A-PE) was purchased from Molecular Probes (Eugene, Oregon). Cells ($2.5–11 \times 10^4$ CNS mononuclear cells or 10^6 splenocytes) were incubated with a predetermined optimal concentration of biotinylated anti-B7 MAb in 2.4G2 supernatant for 30 min at 4°C. Cells were washed in isotonic-buffered saline (Baxter; containing 0.1% NaN_3 and 1.0% normal goat serum) followed by incubation with A-PE and the appropriate FITC-conjugated MAb (anti-CD3, anti-B220, or anti-F4/80) for 30 min at 4°C in the dark. Cells were washed and resuspended in 1 ml isotonic-buffered saline containing 0.1 μg/ml propidium iodide. Data collection and analysis were performed on a FACScan flow cytometer (Becton-Dickinson, Mountain View, California). Sufficient events were collected to assure that at least 4×10^3 large mononuclear cells were analyzed per sample. Nonspecific staining was determined by incubating samples with the appropriately conjugated isotype-matched control MAbs.

MLR Assay

SJL/J splenocytes (4×10^5) isolated from SJL/J mice at different stages of disease were irradiated (2000 rads) and incubated with 100 μg/ml of blocking or control antibodies for 15 min prior to the addition of 2×10^5 purified C57BL/6 T cells. Cultures were incubated at 37°C for 4 days and pulsed with 1 μCi/well of [^{3}H]thymidine for the final 18 hr. Proliferation is presented as the mean cpm of triplicate wells. SEMs were <10%.

Ag-Specific DTH

DTH responses were quantitated using a 24 hr ear swelling assay (McRae et al., 1992). Prechallenge ear thickness was determined using a Mitutoyo model 7326 engineer's micrometer (Schlesinger's Tools, Brooklyn, New York). Immediately thereafter, DTH responses were elicited by injecting 5 μg of peptide (in 10 μl of saline) into the dorsal surface of the ear using a 100 μl Hamilton syringe fitted with a 30 gauge needle. The increase in ear thickness over prechallenge measurements was determined 24 hr after ear challenge. Results are expressed in units of 10^{-4} inches ± SEM. Ear swelling responses were the result of mononuclear cell infiltration and showed typical DTH kinetics (i.e., minimal swelling at 4 hr, maximal swelling at 24–48 hr).

Statistical Analyses

Comparison of the percentage of animals showing clinical relapses between any two groups of mice were analyzed by χ^2 using Fisher's exact probability. Comparisons of the mean day of onset of relapse, mean peak disease severity, and DTH responses between any two groups of mice were analyzed by the Student's t test. p values < 0.05 were considered significant.

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Note Added in Proof

The data cited as "S.G. and R.A.F., unpublished data" in the text has since been published: Guerder, S., Carding, S., and Flavell, R.A. (1995). B7 costimulation is necessary for the activation of the lytic function in cytotoxic T lymphocyte precursors. J. Immunol. *155*, 5167–5174.